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(54) Title: MINK2-MODULATOR OF POTASSIUM CHANNELS

(57) Abstract: This invention generally relates to a novel gene sequence encoding the MinK2 protein, as well as novel methods for the screening of compounds that are agonistic or antagonistic to K^+ activity.

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Mink2 - Modulator of Potassium Channels

This invention was made in part with government support under grants HL-36930, HL-55404, HL-57416 and NS-23877 from the NIH. The government has certain rights in the invention.

FIELD OF THE INVENTION

This invention generally relates to a novel K^+ channel gene named *MinK2*. Additionally, this invention relates to cells and organisms that are made deficient in expression of this gene or made to express additional copies of this gene. Furthermore, this invention relates to drugs screens for compounds that are agonistic or antagonistic to MinK2 or MinK2 influenced K^+ channel activity. Further still, screens for MinK2 intraspecific and interspecific homologs as well as MinK2 associated binding molecules are contemplated.

BACKGROUND

MinK, a potassium channel protein sharing little similarity with other cloned potassium channels, induces very slow voltage-dependent K^+ channel activity in *Xenopus* oocytes expression system (Kaczmarek, L.K. and Blumethal, E.M. "Properties and regulation of the minK potassium channel protein" *Physiol Rev* 77:627-641, 1997). It has only 130 amino acids and a single putative transmembrane domain. The cDNA for MinK was first clone from rat kidney (Takumi, T., *et al.* "Cloning of a membrane protein that induces a slow voltage-gated potassium current" *Science* 242:1042-1045, 1988) and subsequently from neonatal rat heart (Folander, *et al.* "Cloning and expression of the delayed-rectifier IsK channel from neonatal rat heart and diethylstilbestrol-primed rat uterus" *Proc Natl Acad Sci, USA* 87:2975-2979, 1990). These cDNA clones exhibit identical protein sequences, indicating a possible absence of tissue-specific isoforms for this protein. Mouse heart (Honore, E., *et al.* "Cloning, expression, pharmacology and regulation of a delayed rectifier K+ channel in mouse heart" *EMBO J* 10:2805-2811, 1991) and human heart (Krafte, D.S., *et al.* "Electrophisiological properties of a cloned,

human potassium channel expressed in *Xenopus* oocytes" *Biophys J* 61:A378-A378, 1992) MinK also show high sequence identity with the rat MinK protein, suggesting evolutionary conservation of biological function and the likelihood that family members are rare or nonexistent (Wang, W., *et al.* "MinK-KvLQT1 fusion proteins, evidence for multiple stoichiometries of the assembled I_{sK} channel" *J Biol Chem* 273:34069-34074, 1998).

Expression of MinK in *Xenopus* oocytes or in HEK293 cells can induce a unique slowly activating voltage-dependent K^+-selective current that closely resembles native I_{sK}, the slowly activating and non-inactivating K^+ channel current well characterized in heart (Kaczmarek, L.K. and Blumethal, E.M. "Properties and regulation of the minK potassium channel protein" *Physiol Rev* 77:627-641, 1997; Freeman, LC and Kass, R.S. "Expression of a minimal K+ channel protein in mammalian cells and immunolocalization in guinea pig heart" *Circ Res* 73:968-973, 1993). Key experiments in *Xenopus* oocytes suggested that the MinK protein was a regulator of expressed channel activity and not sufficient by itself to form functional I_{sK} channels (Attali, B., *et al.* "The protein IsK is a dual activator of K+ and Cl- channels" *Nature* 365:850-852, 1993; Blumethal, E.M. and Kaczmarek, L.K. "The minK potassium channel exists in functional and nonfunctional forms when expressed in the plasma membrane of Xenopus oocytes" *J Neurosci* 14:3097-3105, 1994). This work has been confirmed based on genetic linkage analysis of an inherited cardiac arrhythmia, the Long QT (LQT1) syndrome and positional cloning strategies (Wang, Q., *et al.* "Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias" *Nat Genet* 12:17-23, 1996; Kass, R.S. "Genetically induced reduction in small currents has major impact" *Circulation* 96:1720-1721, 1997). Co-expression of KvLQT-1, a cardiac K^+ channel linked to LQT1 with MinK, elicits membrane current with properties of I_{sK} (Barhanin, J., *et al.* "K(V)LQT1 and lsK (minK) proteins associate to form the I(Ks) cardiac potassium current" *Nature* 384:78-80, 1996; Sanguinetti, M.C., *et al.* "Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac I(Ks) potassium channel" *Nature* 384:80-83, 1996).

K^+ channels, either functioning or malfunctioning, are implicated in many disease states including cardiac arrhythmias (*e.g.* LQTS: long QT syndrome) (Yang, W-P., *et al.*

"KvLQT1, a voltage-gated potassium channel responsible for human cardiac arrhythmias" *Proc Natl Acad Sci. USA* 94:4017-4021, 1997), hypertension, angina, asthma, diabetes, renal insufficiency, urinary incontinence, irritable colon, epilepsy, cerebrovascular ischemia and autoimmune disease. Such diseases afflict millions of people world wide. For example, cardiac arrhythmias cause about a third of a million deaths each year in the USA alone (Sansom, M.S.P. "Ion channels: Structure of a molecular brake" *Current Biology* 9:R173-R175, 1999). Accordingly, efforts are underway to identify and characterize pharmacological agents that alter the kinetics, gating or formation of K^+ channels. The efficacy of such agents is determined by treating cells with such agents and measuring changes in current across the plasma membrane of the cells. Unfortunately, it is difficult to measure small changes in whether a pharmacological agent alters current flow through a specific K^+ channel due to the limited number of native channels on cells. Additionally, the components of many K^+ channels have not been identified thus making the study of those channels difficult if not impossible. Accordingly, it is desirable to have methods and tools which can be used to examine the K^+ channels on the plasma membrane of cells. It is also desirable to have new research tools that can be used for examining the assembly and synthesis of K^+ channels. For example, the interaction of MinK with the KvLQT1 protein changes inactivation and activation properties of the assembled channels (Tristani-Firouzi, M. *et al.* "Voltage-dependent inactivation of the human K+ channel KvLQT1 is eliminated by association with minimal K+ channel (minK) subunits" *J Physiol (Lond)* 510:37-45, 1998; Tzounopoulos, T., *et al.* "Gating of I(sK) channels expressed in Xenopus oocytes" *Biophys J* 74:2298-2305, 1998), and some experiments have suggested that MinK may affect permeation properties of the channel pore (Romey, G., *et al.* "Molecular mechanism and functional significance of the MinK control of the KvLQT1 channel activity" *J Biol Chem* 272:16713-16716, 1997; Goldstein, S.A.N. and Miller, C. "Site-specific mutations in a minimal voltage-dependent K+ channel alter ion selectivity and open-channel block" *Neuron* 7:403-408, 1991; Tai, K.K., *et al.* "MinK potassium channels are heteromultimeric complexes" *J Biol Chem* 272:1654-1658, 1997; Tai, K.K. and Goldstein, S.A. "The conduction pore of a cardiac potassium channel" *Nature* 391:605-

608, 1998). In this regard, it is likely that MinK may share responsibility for the etiology of certain K^+ channel related diseases. However, the necessary reagents and tools, in many instances, have yet to be discovered.

What is needed is the identification and isolation of molecules and reagents that affect K^+ channel activity thereby allowing for the *in vivo* and *in vitro* screening of compounds that can be used for the treatment of diseases caused by aberrant K^+ activity.

SUMMARY OF THE INVENTION

The present invention relates to novel human (SEQ ID NO:1) and rat (SEQ ID NO:2) gene sequences that encode a K^+ channel regulatory protein. The gene, *MinK2*, is the first new member of this gene family to be discovered in over ten years and only the second member of the family to ever be discovered. *MinK2* has 26% homology at the nucleotide level and 57% homology at the protein level to *MinK1*. Unlike MinK1, which functionally associates with KvLQT1 and is a positive inducer of the current I_{ks}, MinK2 functionally associates with and acts to suppress the currents generated by KvLQT2 and KvLQT3.

The present invention generally comprises a novel, substantially purified oligonucleotide sequence that encodes for the newly discovered gene, *MinK2*. Although the present invention is not limited by any particular mechanism, the expression product of this gene is believed to function as a regulatory protein affecting K^+ channel activity. Unlike the only other known MinK protein, MinK2 does not act to potentiate K^+ activity. Rather, MinK2 acts to suppress K^+ activity. This gene and derivative gene products will allow for methods and tools which can be used to regulate the numbers and types of MinK2/K^+ channels on the plasma membrane of cells and, thus, provide novel reagents and methods for the detection of compounds that are agonistic or antagonistic to MinK2/K^+ channel function.

Although the present invention is not limited to any particular mechanism, it has been determined that expressing polynucleotides that encode MinK2 in host cells, along with polynucleotides that encode the KvLQT2 or KvLQT3 channel proteins, increase the number and activity of KvLQT2 or KvLQT3 channels, respectively, in the plasma

membrane of such cells. Accordingly, MinK2 polynucleotides are useful for making cells that have increased numbers of highly active K^+ channels on the cellular plasma membrane. Such cells are useful model systems for studying the effect of pharmacological agents on K^+ channels, particularly on MinK2/KvLQT2 and MinK2/KvLQT3 channels. In particular, such cells are useful for screening compounds that modulate K^+ fluxing by said channels.

The present invention generally relates to compositions and methods of identifying and testing K^+ channel pathway agonists and antagonists. The present invention is not limited by the method of the employed screen. In one embodiment, the present invention contemplates screening suspected compounds in a system utilizing transfected cell lines or microorganisms. In one embodiment, the cells or microorganisms may be transfected transiently. In another embodiment, the cells may be stably transfected. In yet another embodiment, translation products of the invention may be used in a cell-free assay system. Furthermore, in yet another embodiment, antibodies generated to the translation products of the invention may be used in immunoprecipitation assays. In still another embodiment cell based assays incorporating transfected cells (*e.g.* transiently or stability transfected cells) may be used to screen for K^+ channel agonists and antagonists. And in still another embodiment, transgenic animals may be generated with the transgene contained in a vector containing an inducible, tissue specific promoter or a restrictive promoter such as a metallothione promoter. The present invention also relates to the anti-sense sequences of SEQ ID NO:1 and SEQ ID NO:2, as well as the anti-sense sequences of the transcription products of SEQ ID NO:1 and SEQ ID NO:2. In one embodiment, said sequences are transfected into cells to inhibit the expression of the endogenous MinK2 gene.

The invention also relates to methods to identify other binding partners of the MinK2 gene product. The present invention is not limited to the methods employed to identify MinK2 binding partners. In one embodiment, antibodies generated to translation products of the invention may be used in immunoprecipitation experiments to isolate novel MinK2 binding partners or natural mutations thereof. In another embodiment, the invention may be used to generate fusion proteins (*e.g.* MinK2 fusion proteins) that could

also be used to isolate novel MinK2 binding partners or natural mutations thereof. In yet another embodiment, screens may be conducted using the yeast two-hybrid system using MinK2 as the bait. In yet another embodiment, screens may be conducted using affinity chromatography using MinK2 as the ligand.

The invention also relates to the production of derivatives of the *MinK2* gene such as, but not limited to, mutated gene sequences (and portions thereof), transcription products (and portions thereof), expression constructs, transfected cells and transgenic animals generated from the nucleotide sequences (and portions thereof). The present invention also contemplates antibodies (both polyclonal and monoclonal) to the gene product or nucleic acid aptamers (*i.e.*, an oligonucleotide capable of binding with a target molecule such as an antibody), including the product of mutated genes.

The present invention contemplates using oligonucleotide probes that are complementary to a portion of the MinK2 gene sequence to detect the presence of the MinK2 DNA or RNA. Such probes are preferably between approximately10 and 50 bases and more preferably between approximately 50 and 100 bases. On the other hand, the present invention also contemplates probes complementary to less conserved regions or even unique regions (*e.g.* a portion of the gene having a sequence unique to the MinK2 gene).

In addition, the present invention contemplates a diagnostic wherein, for example, a sample of the DNA of the MinK2 gene sequence is determined (*e.g.* by sequencing) to identify suspected mutations. In such a method, the present invention contemplates isolating the gene from a mixture of DNA. Such isolation can be done using one or more of the probes describes above. For example, the present invention contemplates utilizing oligonucleotides that are complementary to the gene as primers in PCR (*see* U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188, all of which are hereby incorporated by reference). Such primers can be complementary to internal regions of the gene. More preferably, primers can be designed that will hybridize to each end of the gene so that the entire gene can be amplified and analyzed (*e.g.* for mutations).

The present invention also relates to the identification of new homologs of *MinK2* or natural mutations thereof. The present invention is not limited to a particular method

to identify *MinK2* homologs. The present invention contemplates screening for homologs using a variety of molecular procedures. In one embodiment, screens are conducted using Northern and Southern blotting. In another embodiment, screens are conducted using DNA chip arrays composed of *MinK2* DNA sequences for binding complementary sequences. The invention contemplates methods for screening for intra- and inter-specific homologs of MinK2, one method comprising (for example): a) providing in any order: i) extracts from cell suspected of containing said homolog, ii) antibodies reactive to MinK2 and specific for at least a portion of the peptide of MinK2; and b) mixing said antibody with said extract under conditions such that said homolog is detected. The present invention further contemplates a method to screen for homologs of MinK2 comprising: a) extracts from cells suspected of containing said homolog; b) contacting the extract with MinK2; c) detecting said homolog by techniques known to those practiced in the art, for example Western blotting. Polynucleotides containing the MinK2 gene may also be fused in frame to a marker sequence which allows for purification on the MinK2 protein such as the maltose binding protein, which binds to amylose resin, or glutathione, which binds glutathione-S-transferase-coupled resin. Polynucleotides encoding MinK2 protein or MinK2 peptide fragments may also be fused in frame to a marker sequence, such as c-myc, which encodes an eptitope tag that allows for monitoring the intracellular location of MinK2 using commercially available antibodies.

The invention also contemplates novel compositions such as the *MinK2* gene sequence (or portion thereof) inserted into an expression vector or a transfection vector. The invention is not limited to a particular vector. Many commercial vectors are available. Additionally, novel vectors may be made and utilized. The present invention also contemplates a composition comprising said transfection vector transfected into primary cells, a cell line, a microorganism (*e.g.* paramecium) or embryonic cells (*e.g.* *Xenopus* oocytes). The invention is not limited to a particular cell line, cell type or to any particular species from which the cells are derived. The present invention is not limited to a particular transfection method. Many transfection methods are envisioned by the present invention including electroporation, lipofectamine methods, $CaCl_2$ methods (see, generally, Sambrook *et al.* Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold

Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Current Protocols in Molecular Biology (1996) John Wiley and Sons, Inc., N.Y.,) and particle bombardment (Boileau, A.J., *et al.*, "Transformation of *Paramecium tetraurelia* by electroporation or particle bombardment" *J Euk Microbiol* 46:56-65, 1999), which is incorporated herein by reference), all of which are known in the art. The present invention contemplates the use of cDNA or cRNA for transfections. Additionally, the transfection of the MinK2 protein is also contemplated by the present invention. In embodiments where more than one vector or sequence is transfected, the vectors or sequences may be transfected either simultaneously or sequentially. The present invention is not limited by the number of different expression vectors or sequences that may be transfected simultaneously. In one embodiment, vectors expressing KvLQT2 or KvLQT3 K^+ channel subunits are transfected along with the vector encoding the MinK2 gene. In another embodiment, the transfected expression vector encodes for both the MinK2 gene and the KvLQT2 or KvLQT3 subunit. The resulting cells, will have on their surface increased numbers of K^+ channels formed by the exogenous KvLQT2 or KvLQT3 subunits and MinK2.

Another contemplated composition comprises the *MinK2* gene sequence in an appropriate vector used to make a transgenic animal or microorganism. Such *MinK2* gene sequences may be mutated by methods know in the art such that they are loss of function (lof) or gain of function (gof) mutants. Additionally, they may be combined with other gene sequences (the secondary gene sequence) for the purposes of producing a fusion product. The invention is not limited to any specific secondary gene sequence. The secondary gene sequence may be used to permit, for example, the isolation of the gene (*e.g.*, a His tag), the isolation of transcription product or the isolation of translation product. Likewise, said secondary sequence may serve as a marker for identifying or visualizing the vector, the translated RNA or the transcribed protein.

Furthermore, the present invention also contemplates using the above-named sequences and derived products in screening assays. The invention is not limited to any particular screening method. In one embodiment, the invention contemplates drug screens for compounds that are agonistic or antagonistic for MinK2 function. In one embodiment cells (*e.g.* mammalian, *Xenopus* oocytes or paramecium) are transfected

with vectors containing a *MinK2* gene, a complementary DNA (cDNA) or a complementary RNA (cRNA). In another embodiment cells (*e.g.*, rat heart or brain cells) are made defective in *MinK2* expression through homologous recombination (*i.e.*, genetic recombination involving exchange of homologous loci useful in the generation of null alleles (knockouts) in transgenic animals) (See generally, te Riele, H, *et al.*, "Consecutive inactivation of both alleles of the pim-1 protooncogene by homologous recombination in embryonic stem cells" *Nature* 348:649-651, 1990). In one embodiment, the expression vectors are under the control of tissue specific promoters (*e.g.* the metallothione promoter). Cells can be exposed to the compound suspected of altering MinK2 function. The culture can then be exposed to metal ions to activate transcription of the *MinK2* gene and inhibition or enhancement of K^+ channel activity can measured by techniques known to those practiced in the art. The invention is not limited to any particular measurement technique. Various methods are envisioned. For example, K^+ channel activity could be measured by the using the conventional two microelectrode voltage-clamp technique. In another embodiment, the transfection and use of paramecium in said screening assay would allow for the large-scale screening of compounds since chemoattractant methods (*i.e.*, the response of paramecia to various chemicals, *e.g.*, food sources, is related to the rate of ion flux at the cell membrane) may be used to quantitate the effect of the suspected compound on K^+ channel activity.

In one embodiment, the present invention contemplates compositions comprising isolated and purified DNA having an oligonucleotide sequence of SEQ ID NO:1 and SEQ ID NO:2 (or portions thereof). The present invention further contemplates a composition comprising RNA transcribed from such DNA as well as a composition comprising protein translated from transcribed RNA. The protein (or portion thereof) can be used as an antigen and the present invention specifically contemplates an antibody produced from the protein or portion of the protein.

The present invention contemplates that the isolated and purified DNA (*i.e.* having an oligonucleotide sequence of SEQ ID NO:1 and SEQ ID NO:2) can be used to make transgenic organisms. For example, the present invention contemplates both transgenic animals comprising such DNA sequences as well as transgenic microorgan-

isms (*e.g.* paramecium) comprising such DNA sequences. Such transgeneic animals and microorganisms will typically be made using such DNA sequences in operable combination with promoters and enhancers in a transfection vector. The present invention also contemplates such vectors and expression constructs comprising such DNA sequences.

While a variety of screening methods are contemplated. In one embodiment, the present invention contemplates a method to detect K^+ channel agonists and antagonists, comprising: a) providing i) one or more compounds suspected of modulating K^+ channel activity, ii) a first mammalian or paramecium cell line or *Xenopus* oocytes comprising the *MinK2* gene; b) contacting a portion of said cells from said transfected cells with said one or more compounds under conditions such that said compound can enter said cells, so as to create treated portions and untreated portions of cells; and c) comparing the amount K^+ channel activity in said treated portion of cells as compared to said untreated portion of cells.

The present invention contemplates transgenic animals and microorganisms that express increased levels of MinK2 or have the expression of MinK2 diminished or inhibited (*i.e.* gene knock-out animals and microorganisms). Such animals and microorganisms can be made by methods known to those practiced in the art.

DESCRIPTION OF THE FIGURES

Figure 1. Macroscopic current recordings from *Xenopus* oocytes.(A) 2 ng HERG WT + 0.2 ng rat minK2 cRNA, (B)2 ng HERG WT cRNA, (inset) voltage protocol.

Figure 2. Current-voltage relation for HERG WT and HERG WT+ minK2 (2+0.2 ng, n=6).

Figure 3. Steady-state activation and inactivation properties are not different between HERG WT and HERG WT+minK2 K^+channels.

Figure 4. Current activation is not different between HERG WT and HERG WT/minK2 K^+ channels (n=4).

Figure 5. C-type inactivation is not different between HERG WT and HERG WT/minK2 K^+ channels.

Figure 6. Current deactivation of HERG channels was altered by co-expression of minK2. Slow and fast time constants are plotted as a function of membrane potential in (A) and (B), respectively.

Figure 7. Macroscopic current recordings from *Xenopus* oocytes. (A) 7.5 ng KvLQT1-WT+2.5 ng minK cRNA, (B)7.5 ng KvLQT1-WT/2.5 ng+ 5 ng minK2 cRNA, (inset) voltage protocol.

Figure 8. Isochronal current-voltage relations analyzed at the end of the test pulse.

Figure 9. DNA sequence of human MinK2.

Figure 10. DNA sequence of rat MinK2.

Figure 11. Alignment of human and rat MinK1 and MinK2 amino acid sequences.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein "agent", "compound" or "drug" is used herein to denote an inorganic or organic compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues that are suspected of having therapeutic properties. The compound, agent or drug may be purified, substantially purified or partially purified.

As used herein "agonist" refers to molecules or compounds which mimic the action of a "native" or "natural" compound. Agonists may or may not be homologous to these natural compounds in respect to conformation, charge or other characteristics. Thus, agonists may or may not be recognized by, e.g., receptors expressed on cell surfaces. In any event, regardless if the agonist is recognized by a natural compound in a manner similar to a "natural" compound or molecule, the agonist may cause physiologic and/or biochemical changes within the cell, such that the cell reacts to the presence of the agonist in the same manner as if the natural compound was present.

As used herein "antagonist" refers to molecules or compounds which inhibit the

action of a "native" or "natural" compound. Antagonists may or may not be homologous to these natural compounds in respect to conformation, charge or other characteristics. Thus, antagonists may be recognized by the same or different receptors or molecules that are recognized by an agonist. Antagonists may have allosteric effects which prevent the action of an agonist (*e.g.*, by modifying a DNA adduct, or antagonists may prevent the function of the agonist (*e.g.*, by blocking a DNA repair molecule).

As used herein "exogenous" means that the gene encoding the protein is not normally expressed in the cell. Additionally, "exogenous" refers to a gene transfected into a cell to augment the normal (*i.e.* natural) level of expression of that gene.

As used herein "anti-sense" shall be defined as a nucleotide sequence that is complementary to another single strand of DNA or RNA. Said complementation is typically at least 50%, more typically said complementation is greater than 75%, even more typically said complementation is greater than 90%.

"Gain of function" (gof) shall be defined as all modifications to an oligonucleotide that, when that oligonucleotide is transfected into a host organism and translated into a peptide, that peptide will function with increased efficiency as compared to the wild type peptide when the gene or gene product is induced to function whether that induction be continuous or non-continuous. It may, in effect, function as an augmenter of the natural gene if the natural gene is present and functional in the host into which the gof oligonucleotide was transfected, or it may add that function to the host if the natural gene is not present or is non-functional.

"Loss of function" (lof) shall be defined as all modifications to an oligonucleotide that, when that oligonucleotide is transfected into a host organism and translated into a peptide, that peptide will function with decreased efficiency as compared to the wild type peptide when the gene or gene product is induced to function whether that induction be continuous or non-continuous. It may, in effect, function as a diminisher of natural gene function if the natural gene is present and functional in the host into which the lof oligonucleotide was transfected, or may negatively interfere with processes in the host if the natural gene is not present or is non-functional.

As used herein, the term "purified" or "to purify" refers to the removal of contami-

nants from a sample. The present invention contemplates purified compositions (discussed above).

As used herein, the term "partially purified" refers to the removal of a moderate portion of the contaminants of a sample to the extent that the substance of interest is recognizable by techniques known to those skilled in the art as accounting for an amount of the mixture greater than approximately 5% of the total.

As used herein, the term "substantially purified" refers to the removal of a significant portion of the contaminants of a sample to the extent that the substance of interest is recognizable by techniques known to those skilled in the art as the most abundant substance in the mixture.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid. In one embodiment, the present invention contemplates "functional portions" of a protein. Such portions are "functional" if they contain a binding region (i.e. a region having affinity for another molecule) and such binding can take place (i.e. the binding region functions, albeit with perhaps lower affinity than that observed for the full-length protein). Such "functional portions" of the gene product are typically greater than approximately 10 amino acids in length, and more typically greater than approximately 50 amino acids in length, and even more typically greater than 100 amino acids in length. "Functional portions" may also be "conserved portions" of the protein. The alignment of the various gene products permit one skilled in the art to select conserved portions of the protein (i.e. those portions in common between two or more species) as well as unconserved portions (i.e. those portions unique to two or more species). The present invention contemplates conserved portions 10 amino acids in length or greater, and more typically greater than 50 amino acids in length. See Figure 11 for the alignment of human and rat MinK1 and MinK2 amino acid sequences.

"In operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a

desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

"Expression vector" shall be defined as a sequence of DNA or RNA, in operable combination that is used to transfect a cell or cells. The sequence may be single or double stranded.

"Patient" shall be defined as a human or other animal, such as a farm animal, guinea pig or mouse and the like, capable of having diseases resulting from adverant K^+ channel activity.

GENERAL DESCRIPTION OF THE INVENTION

Voltage-gated K^+ channels (K^+) are important in the physiology of both excitable and nonexcitable cells. The diversity in K^+ currents is reflected in multiple K^+ channel genes whose products may assemble as multisubunit heteromeric complexes. Given the fundamental importance and diversity of K^+ channels, surprisingly little is known regarding the *in vivo* cellular mechanisms regulating their synthesis, assembly and metabolism. The present invention will help to elucidate these mechanisms and also permit the screening of compounds that are antagonistic or agonistic to MinK2-associated K^+ channel function.

Although the present invention is not limited by any particular mechanism, it is currently believed that delayed K^+ rectifier channels initiate the repolarization that terminates the plateau phase of the action potential. The delayed rectifier K^+ current is the addition of two components rapidly activating one, which is called I_{Kr}, and a very slowly activating current called I_{Ks} (Sanguinetti, M.C. and Jurkiewicz, N.K. "Two components of cardiac delayed rectifier K+ current. Differential sensitivity to block by class III antiarrhythmic agents" *J Gen Physiol* 96:195-215, 1990). Cardiac arrhythmias, based on abnormal repolarization, are visualized as a prolonged QT interval on an electrocardiogram. Congenital long QT (LQT) is an inherited disease characterized by prolonged ventricular repolarization that causes syncope and sudden death due to ventricular arrhythmia (Wang, Q., *et al.* "Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias" *Nat Genet* 12:17-23, 1996). The LQT

syndrome is genetically heterogeneous with at least four chromosomal loci (LQT1 to LQT4) implicated in the disease. One of them, the LQT2 locus, corresponds to mutations in the HERG gene that encodes the rapidly activating delayed rectifier K^+ channel generating I_{kr}. Another one, the LQT1 locus, encodes a K^+ channel protein, KvLQT1, that associates with another small transmembrane protein known as MinK1, to generate the slowly activating K^+ channel I_{Ks} (Barhanin, J., *et al.* "K(V)LQT1 and lsK (minK) proteins associate to form the I(Ks) cardiac potassium current" *Nature* 384:78-80, 1996; Sanguinetti, M.C., *et al.* "Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac I(Ks) potassium channel" *Nature* 384:80-83). Expression of I_{Ks} is not limited to the heart. KvLQT1 as well as MinK are also expressed in many other organs such as kidney and the *stria vascularis* of the inner ear (Marcus, D.C. and Shen, Z. "Slowly activating voltage-dependent K+ conductance is apical pathway for K+ secretion in vestibular dark cells" *Am J Physiol* 267:C857-C864, 1994). Some human mutations of the KvLQT1 gene lead to the Jervell-Lange-Nielsen ayndrome (Neyroud, N. *et al.* "A novel mutation in the potassium channel gene KVLQT1 causes the Jervell and Lange-Nielsen cardioauditory syndrome" *Nat Genet* 15:186-189, 1997). Patients suffering from this syndrome not only exhibit a long QT wave interval but also profound deafness from birth. On the other hand, mice carrying a null mutation on the MinK gene also display profound inner ear dysfunction associated with drastically altered K^+ secretion into the endolymph of the inner ear leading to hair cell degeneration (Vetter, D.E., *et al.* "Inner ear defects induced by null mutation of the isk gene" *Neuron* 17:1251-1264, 1996). Thus the KvLQT1/MinK1 assembly forms a K^+ channel that has a key secretory role in ventricular repolarization and a key secretory role in the control of endolymph homeostasis associated with normal hearing. The present invention will allow for the determination of the mechanism of the functions of the MinK2/KvLQT2 and MinK2/KvLQT3 association and allow for the screening of compounds to treat associated diseases.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below

are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see, generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Current Protocols in Molecular Biology (1996) John Wiley and Sons, Inc., N.Y., which are incorporated herein by reference) which are provided throughout this document.

Oligonucleotides can be synthesized on an Applied BioSystems oligonucleotide synthesizer [for details see Sinha *et al.*, Nucleic Acids Res. 12:4539 (1984)], according to specifications provided by the manufacturer. Complementary oligonucleotides are annealed by heating them to 90°C in a solution of 10 mM Tris-HCl buffer (pH 8.0) containing NaCl (200 mM) and then allowing them to cool slowly to room temperature. For binding and turnover assays, duplex DNA is purified from native polyacrylamide (15% w/v) gels. The band corresponding to double-stranded DNA is excised and soaked overnight in 0.30 M sodium acetate buffer (pH 5.0) containing EDTA (1 mM). After soaking, the supernatant is extracted with phenol/chloroform (1/1 v/v) and precipitated with ethanol. DNA substrates are radiolabeled on their 5'-OH group by treatment with [g-^{32}P]ATP and T4 polynucleotide kinase. Salts and unincorporated nucleotides are removed by chromatography on Sephadex G columns.

Assays for detecting the ability of agents to inhibit or enhance K^+ channel activity provide for facile high-throughput screening of agent banks (e.g., compound libraries, peptide libraries, and the like) to identify antagonists or agonists. Such K^+ antagonists and agonists may be further developed as potential therapeutics and diagnostic or prognostic tools for diverse types of neurological and muscular diseases, as well as cardiac arrhythmias (*e.g.* LQTS: long QT syndrome), hypertension, angina, asthma, diabetes, renal insufficiency, urinary incontinence, irritable colon, epilepsy, cerebrovascular ischemia and autoimmune disease. Likewise, the *MinK2* gene, and modifications thereof,

may be useful in gene therapy. For example, the incorporation of the *MinK2* gene sequence into cells in context of tissue specific or inducible promoters is contamplated to be useful in the treatment of hereditary diseases.

1. Screens to identify Agonists of Antagonists of K^+ Channel Activity

There are several different approaches contemplated by the present invention to look for small molecules that specifically inhibit or enhance K^+ channel activity. One approach is to transfect expression constructs (vectors) comprising the MinK2 gene into cells and measure changes in the rate of K^+ flux as compared to controls after the cells have been exposed to the compound suspected of modulating MinK2 activity. Cells may be transiently transfected or stably transfected with the construct under control of an inducible or temperature sensitive promoter. Another embodiment is to transfect cRNA for the MinK2 protein. In yet another embodiment, cRNA is transfected simultaneously with cRNA encoding a Kv channel protein. Other embodiments would include translation of the invention and purification of the peptide. The purified peptide could then be used as a substrate in a cell-free assay. Furthermore, transgenic animals and stably transfected cell lines could be produced allowing for *in vivo* assays to be conducted.

A. *In vitro* Assays

i. Transfection Assays

Transfection assays allow for a great deal of flexibility in assay development. The wide range of commercially available transfection vectors will permit the expression of the invention in a extensive number of cell types. In one embodiment, cells are transiently transfected with an expression construct comprising, in operable combination, the MinK2 gene and an inducible promoter allowing for the initiation of translation and transcription when needed. Cells are exposed to the agent suspected of modulating K^+ activity, MinK2 expression is initiated and K^+ channel activity is measured. Rates of K^+ flux in cells treated with said compound are compared to rates in cells that are untreated. Rates of K^+ fluxing are quantitated by any of a number of ways reported in the literature and known to those practiced in the art.

In another embodiment, stably transfected cells lines are employed. The use of an

inducible promoter or temperature sensitive promoter can be utilized in these systems. Screening assays for compounds suspected of modulating K^+ channel activity are conducted in the same manner as with the transient transfection assays. Using stably transfected cell lines, however, allows for greater consistency between experiments and allow for inter-experimental comparisons.

In order to test the stimulatory or inhibitory effect of a compound, particularly a pharmacological agent, on the flow of a current through Kv channels, it is desirable to have a model system comprising a population of cells that have increased numbers of Kv channels on their cellular plasma membrane. Such model system is especially suitable for measuring small changes in current flow. Such model systems are prepared by coinjecting into host cells cDNA or cRNA molecules encoding MinK2 and for the Kvα subunit. The encoding regions for MinK2 and for the Kvα subunit may be on separate cDNA or cRNA molecules. Preferably, the Kvα subunit is an exogenous Kvα subunit, *i.e.*, the Kvα subunit is not normally expressed in the cell. Such model systems are especially useful for monitoring the effect of a compound on a particular Kv channel, *i.e.*, the Kv channel formed by assembly of a plurality of the exogenous Kvα subunits. Thereafter, the cells are cultured for a time and under conditions which permit transformation of the host cells, *i.e.*, expression of the coinjected cDNA or cRNA molecules and assembly of Kv channels comprising the corresponding Kvα subunits.

The compound, which has been dissolved in a suitable carrier, is added to the culture medium of a test population of transformed host cells. Preferably, a plurality of concentrations of the compound are added to a corresponding plurality of test populations. The compound is also added to the culture medium of a control population of cells that have not been transformed, *i.e.*, cRNA or cDNA molecules encoding MinK2 and the Kvα subunit are not transfected into the cell. Thereafter, whole cell currents are measured using conventional techniques, such as, for example, using a two microelectrode voltage-clamp technique and the gigaseal patch clamp technique. A difference between whole cell currents in the control population and the test population is indicative of a stimulatory or inhibitory effect of the compound on the Kv channels formed by the exogenous Kvα subunit. Such measurements are also used to determine the effective com-

pound dosage.

B. *In Vivo* Assays

i. Transgenic Animal Assays

In one embodiment, transgenic animals are constructed using standard protocols, including homologous recombination (*i.e.*, genetic recombination involving exchange of homologous loci useful in the generation of null alleles (knockouts) in transgenic animals) (See generally, te Riele, H, *et al.*, "Consecutive inactivation of both alleles of the pim-1 protooncogene by homologous recombination in embryonic stem cells" *Nature* 348:649-651, 1990). The MinK2 gene may be placed under the control of a tissue specific promoter or inducible promoter. The generation of transgenic animals will allow for the creation of model systems to investigate the numerous diseases associated with aberrant K^+ channel activity which may provide the means for determining the physiology of the disease or its treatment.

ii. Paramecium Based Assays

In yet another embodiment, paramecium are transfected with the *MinK2* gene by methods known to those in the art (*e.g.* electroporation or particle bombardment; Boileau, A.J., *et al.*, "Transformation of *Paramecium tetraurelia* by electroporation or particle bombardment" *J Euk Microbiol* 46:56-65, 1999), which is incorporated herein by reference). Said transfected paramecium are then exposed to compounds suspected of modulating K^+ channel activity. Rates of K^+ flux in the paramecium are measured by chemosensory assays known in the art (Fraga, D., *et al.*, "Introducing antisense oligodeoxynucleotides into *Paramecium* via electroporation" *J Euk Microbiol* 45:582-588, 1998) and compared to rates of K^+ flux in untreated paramecium.

2. Screens to Identify MinK2 Binding Partners

A. *In vitro* Assays

There are several different approaches to identifying MinK2 interactive molecules or binding partners. Techniques that may be used are, but not limited to, immunoprecipitation of MinK2 with antibodies generated to the translation product of the invention. This would also bring down any associated bound proteins, *i.e.* proteins in the

cell with affinity for the MinK2 polypeptide. Another method is to generate fusion proteins comprising MinK2 connected to a generally recognized pull-down protein such as glutathione S-transferase (GST). Bound proteins can then be eluded and analyzed. Yet another method is to bind MinK2 to a solid support and expose the bound MinK2 to cell extracts suspected of containing an MinK2 interactive molecule or binding partner.

i. Immunoprecipitation

After the generation of antibodies to MinK2, cells expressing transfected MinK2 are lysed and then incubated with one of the antibodies. Antibodies interact with the bound MinK2 and any associated proteins can then be pulled down with protein-A Sepharose or protein-G Sepharose beads, using standard techniques. Where yeast binding partners are sought, yeast cells are lysed. Antibodies may be generated to MinK2 by methods known to those practiced in the art. For example, polyclonal antibodies may be generated by injection of the protein of interest, or portion thereof, in combination with an adjavent (*e.g.*, Freud's adjavent), into an animal such as a rabbit or mouse. Antibody titer can be determined by, for example, Octerlony assay and antibodies can be isolated from the blood by, for example, protein Sephorose-G column chromatography. Likewise, monoclonal antibodies can be generated by fusion of an antibody producing B cell line and a melanoma cell line. After selection of clonal cultures that produce the desired antibody, antibody may be purified from culture supernatant (see generally, Kohler, G. and Milstein, C. "Continuous cultures of fused cells secreting antibody of predefined specificity" *Nature* 256:495-497, 1975).

ii. Fusion Protein Pull-down

A method similar to immunoprecipitation is to construct fusion proteins of the mutant and wild type MinK2 and glutathione S-transferase (GST). The GST-MinK2 fusion proteins are then incubated with cell extracts and then removed with glutathione Sepharose beads. Any bound, MinK2-associated proteins are then characterized.

B. *In Vivo* Assays

i. Yeast Two-hybrid System

The yeast two-hybrid system identifies the interaction between two proteins by reconstructing active transcription factor dimers (Chien, C.T., *et al.* "The two-hybrid

system: a method to identify and clone genes for proteins that interact with a protein of interest" *Proc Natl Acad Sci, USA* 88:9578-9582, 1991). The dimers are formed between two fusion proteins, one of which contains a DNA-binding domain (DB) fused to the first protein of interest (DB-X, where X will be MinK2) and the other, an activation domain (AD) fused to the second protein of interest (AD-Y, where Y will be a candidate MinK2-binding protein encoded by cDNA from a commercially available library). The DB-X:AD-Y interaction reconstitutes a functional transcription factor that activates chromosomally-integrated reporter genes driven by promoters containing the relevant DB binding sites. Large cDNA libraries can be easily screened with the yeast-two hybrid system. Yeast cDNA libraries are commercially available. Standard molecular biological techniques can be employed to isolate and characterize the interacting protein.

3. Screens to Identify MinK2 Homologs

Standard molecular biological techniques can be used along with the reagents of the present invention to identify MinK2 homologs in various species. For example, preferred embodiments may included, but are not limited to, DNA-DNA hybridization techniques (e.g. Southern blots) and DNA-RNA hybridization techniques (e.g. Northern blots). Additional techniques may include, for example, immunoscreening of proteins made from library stocks by antibodies generated from the invention. The present invention also contemplates a number of approaches including, but not limited to, immunoprecipitation and affinity purification of cell and tissue extracts and immunoscreening of proteins and glycoproteins translated from DNA and RNA library stocks. Furthermore, hybridization screens of RNA and DNA library stocks could be accomplished using RNA and DNA sequences reverse engineered from isolated MinK2 protein.

EXPERIMENTAL

Materials and Methods

Cloning of MinK2. NCBI database searches identified EST D85797 as a gene fragment showing about 50% homology to MinK (KCNE1). Full length human (SEQ ID NO:1) and rat (SEQ ID NO:2) sequences of this new gene were isolated with PCR-based

methods. Full length sequences of rat MinK2 and human MinK2 were sub-cloned into a pSP64 (Promega) based vector for expression in *Xenopus* oocytes. cRNAs were prepared using the mMessage mMachine in vitro transcription kit (Ambion) and SP6 polymerase. Production and size of full length RNAs was verified by denaturing agarose gel electrophoresis. MinK2 cRNA was co-injected together with HERG WT(SEQ ID NO:3) and with KvLQT1 (SEQ ID NO:4)/MinK (SEQ ID NO:5) cRNAs into stage V-VI *Xenopus* oocytes.

Electrophysiology. Currents were recorded 2 to 7 days after injection using a Dagan 8500 two-electrode voltage-clamp amplifier. Current and voltage electrodes were filled with 3 M KCL and had resistances of about 1 MOhm. The composition of the bath solution used to perfuse oocytes was (mmol/L); NaCl 96, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 5 (pH 7.4). In current recordings of KvLQT-WT/MinK +MinK2 $CaCl_2$ was reduced to 0.1 or 0.5 mM to suppress endogenous Ca^{2+}-activated Cl^- currents. No leak subtraction was used. All measurements were performed at room temperature (20-22°C). pClamp software (Axon Instruments) was used for the generation of voltage-clamp protocols and for data acquisition. All data are expressed as mean +/-S.E.M.

Example 1

Macroscopic current recordings were taken from *Xenopus* oocytes injected with 2 ng HERG WT + 0.2 ng rat minK2 cRNA (Figure 1A) or with 2 ng HERG WT cRNA (Figure 1B). Inset: voltage protocol; holding potential, -85 mV, test pulses, from –100 to +60 mV with 10 mV increments; return potential –90 mV. Recordings were made 2 days after injection.

Example 2

Figure 2 shows current-voltage relation for HERG WT (2 ng cRNA, mean values from 6 oocytes, solid circles) and HERG WT+ minK2 (2+0.2 ng, n=6, open squares). Currents were elicited with voltage protocol shown in inset to Figure 1. Amplitudes were analyzed at the end of depolarizing voltage commands. A single batch of oocytes was used for panel in Figure 2. Recordings were made 2 days after injection.

Example 3

Steady-state activation and inactivation properties are not different between HERG WT and HERG WT+minK2 K^+channels. Steady state activation was analyzed by plotting tail current amplitudes at –90 mV against test potentials (in recordings as shown in Figure 1). Steady-state inactivation was assessed with brief hyperpolarizing voltage steps from +20 mV; plotted are normalized peak currents on return to +20 mV. Data were fitted with Boltzmann equations of the form: $y=1/(1+\exp[(V_h-V)/k])$, where V_h is the half activation potential and k is the slope factor. The values of V_h for inactivation curves were –44.9 +/-0.6 mV for WT (Figure 3, closed triangles) and –53.1+/-0.5 mV for HERGWT/minK2 (Figure 3, open triangles) (n=6-7). The values of V_h for activation curves were –17.3+/-0.1 mV and –14.9+/-0.2 mV for WT (Figure 3, closed circles) and WT/minK2 (Figure 3, open squares), respectively (n=6-8). Furthermore, as shown in Figure 4, current activation is not different between HERG WT and HERG WT/minK2 K^+ channels (n=4). Currents were evoked by an envelope of tails protocol from a holding potential of –85 mV and test pulses of variable duration to 0 mV. Tail currents were analyzed on return to –100 mV, normalized and plotted vs. test pulse duration (n=4). Additionally, as shown in Figure 5, C-type inactivation is not different between HERG WT and HERG WT/minK2 K^+ channels. To assess C-type inactivation channels were opened and inactivated at +40 mV, then recovered from inactivation with a 22.5 ms pre-pulse at-100 mV, and re-inactivated at potentials between –40 and +40 mV. Re-inactivation time courses were fitted with monoexponential functions. Resulting time constants are shown as a function of membrane potential for HERG WT (Figure 5, solid circles) and HERG WT/minK2 (Figure 5, open squares, n=6).

Example 4

Current deactivation of HERG channels was altered by co-expression of minK2. Channels were activated by 700 ms test pulses to +20 mV (holding potential, –80 mV). Current deactivation was assessed by 9 second voltage steps between –40 and –120 mV. Deactivating tail currents were fitted with double-exponential functions in between –70 and –40 mV. Mono-exponential functions were sufficient at potentials more negative

than –80 mV. Slow and fast time constants are plotted as a function of membrane potential in Figure 6A and Figure 6B, respectively.

Example 5

Figure 7 shows macroscopic current recordings from *Xenopus* oocytes injected with 7.5 ng KvLQT1-WT+2.5 ng minK cRNA (Figure 7A) and 7.5 ng KvLQT1-WT/2.5 ng+ 5 ng minK2 cRNA (Figure 7B). Inset: voltage protocol; holding potential, -90 mV, 2400 ms test pulses, from –100 to +60 mV with 20 mV increments; return potential, –120 mV. Figure 8 shows isochronal current-voltage relations analyzed at the end of the test pulse for KvLQT-WT/minK (7.5 ng/2.5 ng cRNA, mean values from 4 oocytes, solid squares), KvLQT1-WT/minK+2.5 or 5 ng minK2 cRNA (2.5 ng minK2, n=5, open circles; 5 ng minK2 cRNA, n=5, open triangles). MinK2 exerts dominant negative effect. Single batch of oocytes used. All recordings made 2 days after cRNA injection.

It should be clear from the above that the compound and methods reported here allows for the screening of compounds that are agonistic or antagonistic for potassium channel activity.

CLAIMS

1. A composition comprising purified DNA selected from SEQ ID NO:1 and SEQ ID NO:2.
2. RNA transcribed from the DNA of claim 1.
3. Protein translated from the RNA of claim 2.
4. Antibodies produced from the protein of claim 3.
5. Expression constructs comprising the DNA of claim 1.
6. A transgenic animal comprising DNA of claim 1.
7. A method to detect *MinK2* agonists and antagonists, comprising:

a) providing i) one or more compounds suspected of modulating *MinK2* activity, ii) a cell line comprising the *MinK2* gene;

b) contacting a portion of said cells from said cell line with said one or more compounds under conditions such that said compound can enter said cells, so as to create treated portions and untreated portions of cells; and

c) comparing the amount of potassium channel activity of said treated cells with the amount of potassium channel activity of said untreated cells.

8. A method to detect *MinK2* agonists and antagonists, comprising:

a) providing i) one or more compounds suspected of modulating *MinK2* activity, ii) a cell line or transfected cells comprising the *MinK2* gene and one gene selected from the a group comprising *KvLQT2* and *KvLQT3*;

b) contacting a portion of said cells from with said one or more compounds under conditions such that said compound can enter said cells, so as to create treated portions and untreated portions of cells; and

c) comparing the amount of potassium channel activity of said treated cells with the amount of potassium channel activity of said untreated cells.

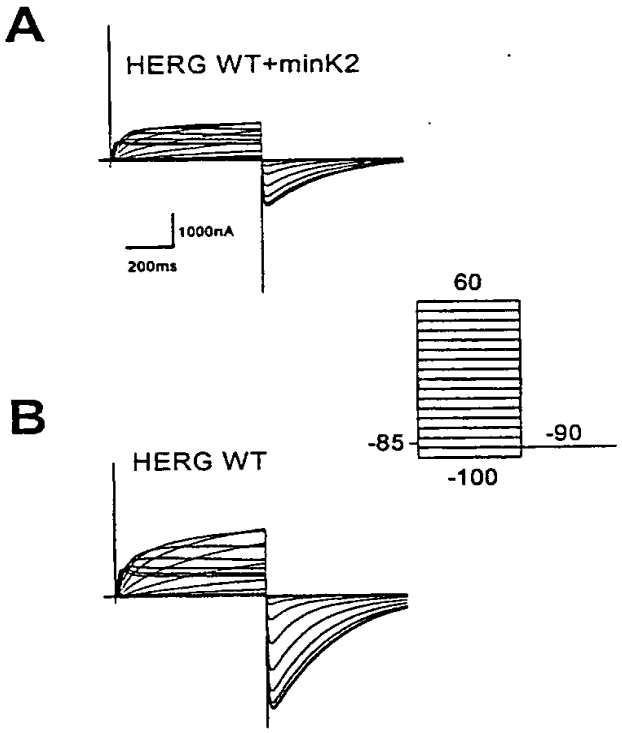

Figure 1

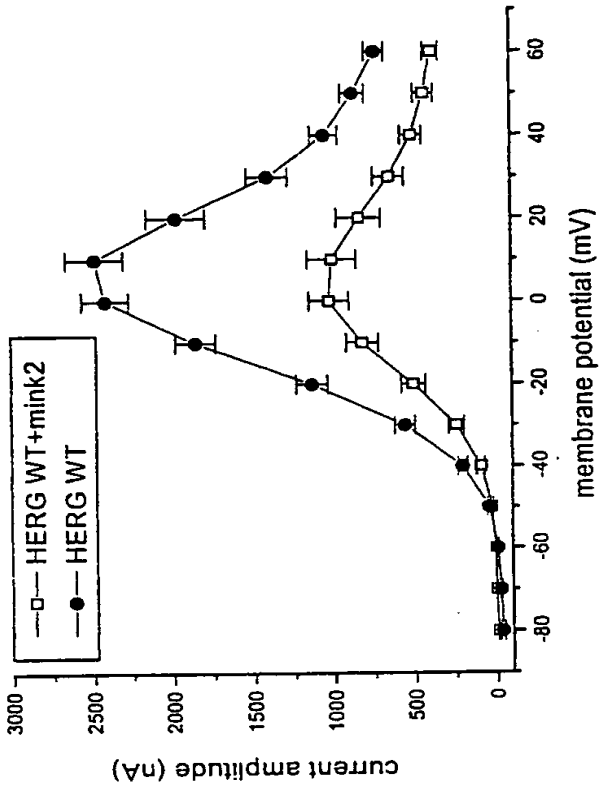
HERG WT+mink2
HERG WT
current amplitude (nA)
membrane potential (mV)
3000
2500
2000
1500
1000
500
0
60
40
20
0
-20
-40
-60
-80

Figure 2

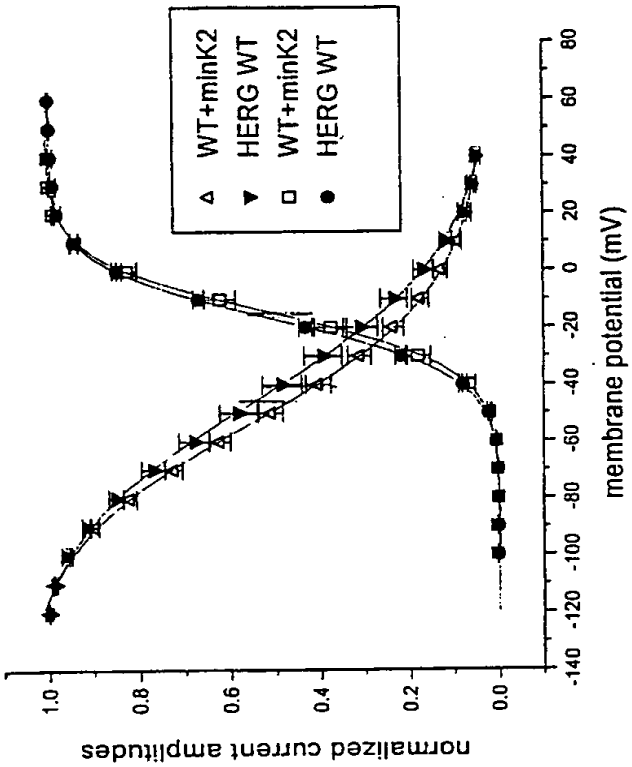

Figure 3

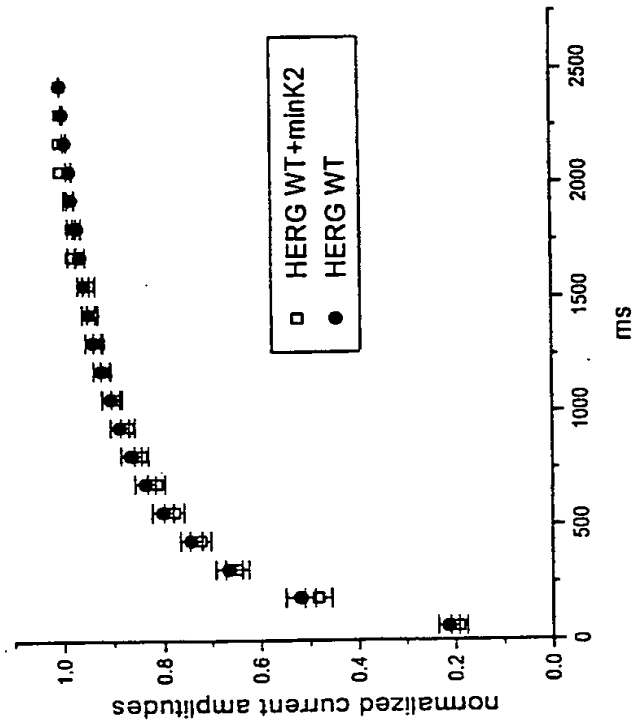
HERG WT+minK2
HERG WT
normalized current amplitudes
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
2500
ms

Figure 4

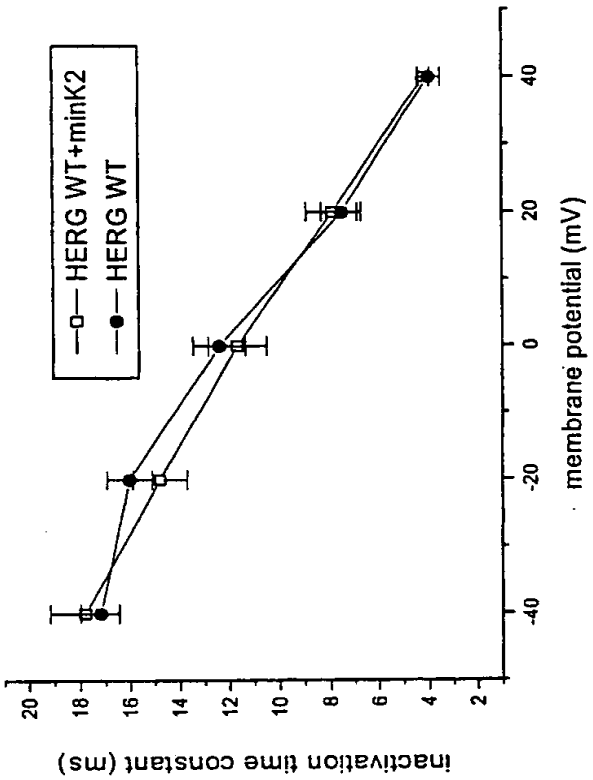

Figure 5

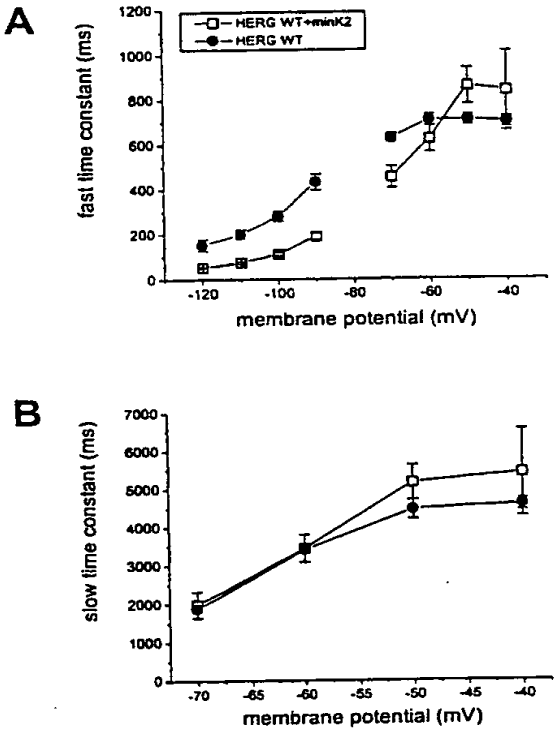
A
HERG WT+minK2
HERG WT
fast time constant (ms)
1200
1000
800
600
400
200
0
-120
-100
-80
-60
-40
membrane potential (mV)
B
slow time constant (ms)
7000
6000
5000
4000
3000
2000
1000
0
-70
-65
-60
-55
-50
-45
-40
membrane potential (mV)

Figure 6

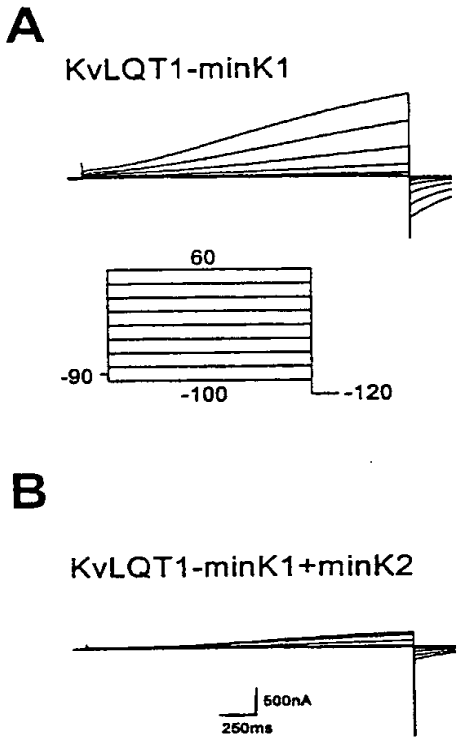

Figure 7

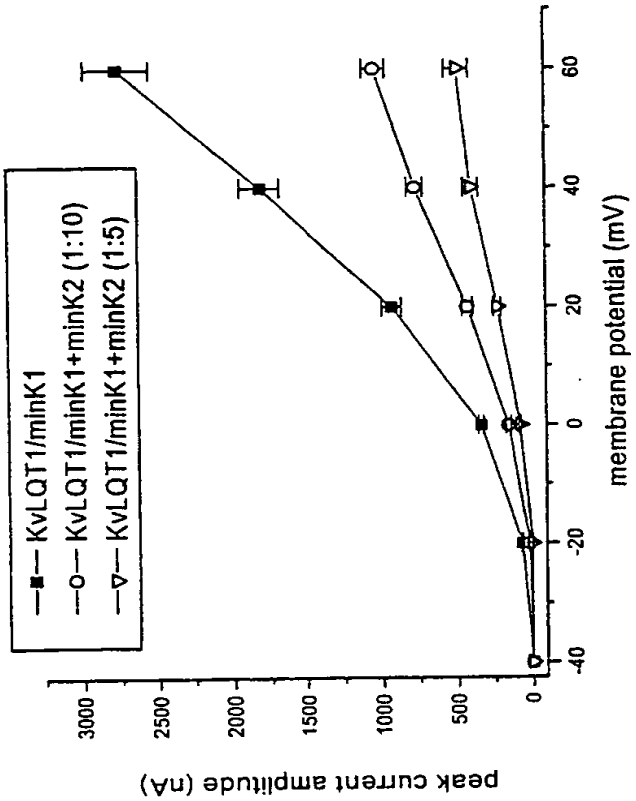
KvLQT1/minK1
KvLQT1/minK1+minK2 (1:10)
KvLQT1/minK1+minK2 (1:5)
peak current amplitude (nA)
membrane potential (mV)
3000
2500
2000
1500
1000
500
0
-40
-20
0
20
40
60

Figure 8

(Linear) MAP of: humanmink2

```
   atgtctactttatccaatttcacacagacgctggaagacgtcttccgaaggatttttatt
 1 ---------+---------+---------+---------+---------+---------+ 60

   M  S  T  L  S  N  F  T  Q  T  L  E  D  V  F  R  R  I  F  I  -

   acttatatggacaattggcgccagaacacaacagctgagcaagaggccctccaagccaaa
61 ---------+---------+---------+---------+---------+---------+ 120

   T  Y  M  D  N  W  R  Q  N  T  T  A  E  Q  E  A  L  Q  A  K  -

   gttgatgctgagaacttctactatgtcatcctgtacctcatggtgatgattggaatgttc
121 ---------+---------+---------+---------+---------+---------+ 180

   V  D  A  E  N  F  Y  Y  V  I  L  Y  L  M  V  M  I  G  M  F  -

   tctttcatcatcgtggccatcctggtgagcactgtgaaatccaagagacgggaacactcc
181 ---------+---------+---------+---------+---------+---------+ 240

   S  F  I  I  V  A  I  L  V  S  T  V  K  S  K  R  R  E  H  S  -

   aatgacccctaccaccagtacattgtagaggactggcaggaaaagtacaagagccaaatc
241 ---------+---------+---------+---------+---------+---------+ 300

   N  D  P  Y  H  Q  Y  I  V  E  D  W  Q  E  K  Y  K  S  Q  I  -

   ttgaatctagaagaatcgaaggccaccatccatgagaacattggtgcggctgggttcaaa
301 ---------+---------+---------+---------+---------+---------+ 360

   L  N  L  E  E  S  K  A  T  I  H  E  N  I  G  A  A  G  F  K  -

   atgtccccctga
361 ----------+-- 372

   M  S  P  *  -
```

Figure 9

(Linear) MAP of: ratmink2

```
   ATGACCACTTTAGCCAACTTGACGCAGACCCTGGAGGATGCCTTCAAAAAGGTTTTCATT
 1 ---------+---------+---------+---------+---------+---------+ 60

   M  T  T  L  A  N  L  T  Q  T  L  E  D  A  F  K  K  V  F  I  -

   ACTTATATGGACAGCTGGAGGAGGAACACAACAGCCGAACAACAGGCGCTCCAGGCCAGA
61 ---------+---------+---------+---------+---------+---------+ 120

   T  Y  M  D  S  W  R  R  N  T  T  A  E  Q  Q  A  L  Q  A  R  -

   GTGGATGCCGAGAACTTCTACTACGTCATCCTGTACCTCATGGTGATGATCGGCATGTTC
121 ---------+---------+---------+---------+---------+---------+ 180

   V  D  A  E  N  F  Y  Y  V  I  L  Y  L  M  V  M  I  G  M  F  -

   GCCTTCATCGTGGTGGCCATCCTGGTGAGCACGGTGAAGTCGAAGCGGCGGGAGCACTCC
181 ---------+---------+---------+---------+---------+---------+ 240

   A  F  I  V  V  A  I  L  V  S  T  V  K  S  K  R  R  E  H  S  -

   CAGGACCCGTACCACCAGTaCaTCGTGGAGGATTGGCAGCAGAAGTATAGGAGTCAGATC
241 ---------+---------+---------+---------+---------+---------+ 300

   Q  D  P  Y  H  Q  Y  I  V  E  D  W  Q  Q  K  Y  R  S  Q  I  -

   TtGCATCTGGAAGACTCCAAGGCCAcCATCCATGAgAACCTGGGGGCGACGGGGTTCACA
301 ---------+---------+---------+---------+---------+---------+ 360

   L  H  L  E  D  S  K  A  T  I  H  E  N  L  G  A  T  G  F  T  -

   gTGTCACCCTGA
361 ----------+-- 372

   V  S  P  *  -
```

Figure 10

Alignment of rat and human minK2 with rat and human minK

```
RatminK2    MtTLaNLTQt LEDaFkkVFI tYMDSwRrnT TAEqqaLqAr vd.....AEn
humanminK2  MsTLSNFTQt LEDvFrrIFI tYMDnwRQnT TAEqeaLqAk vd.....AEn
ratminK     -MaLSNsTtv L..... pFLaSlWQeT depgGnMsAd laRRSqlrDd
humanminK   -MiLSNtTav t..... pFLtklWQeT vqqgGnMs.g laRRSprssd
Consensus   ---L-N-T-- --------- ---------T ---------- ----------

ratminK2    FyyviLYlMv miGmFaFivv aILvStVkSK rrEHSqDPYh qYIveD.WQq
humanminK2  FyyviLYlMv miGmFsFiiv aILvStVkSK rrEHSNDPYh qYIveD.WQE
ratminK     skleaLYiLm vlGfFGFftl gIMlSyIrSK klEHShDPFn vYIesDAWQE
humanminK   gkleaLYvLm vlGfFGFftl gIMlSyIrSK klEHSNDPFn vYIesDAWQE
Consensus   -----LY--- --G-F-F--- -I--S---SK --EHS-DP-- -YI--D-WQ-

ratminK2    K....YrsqI Lh.....l EdSKAtiheN lgatgFtvSP *
humanminK2  K....YksqI Ln.....l EeSKAtiheN igaagFKmSP *
ratminK     KgKAlFQarV LESFRaCYVi EnqaAveqpa ThlPeLKPls .
humanminK   KdKAyvQarV LESYRsCYVv EnhlAieqpN ThlPetKPSP .
Consensus   K--------- L--------- E---A----- ---------- -
```

Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/22799

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL : 536/23.5; 435/320.1, 325, 455; 530/350, 387.1, 800/13
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/320.1, 325, 455; 530/350, 387.1, 800/13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ABBOTT et al. MiRP1 forms I_{kr} potassium channels with HERG and is associated with cardiac arrhythmia. Cell. 16 April 1999, Vol. 97, pages, 174-187, whole document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report 05 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No.	Authorized officer PETER PARAS JR. Telephone No. (703) 308-8340

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/22799

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C07H 21/04; C12N 5/00, 5/02, 15/00, 15/09, 15/63, 15/70, 15/74, 15/85, 15/87; C07K 1/00, 14/00, 16/00, 17/00; A01K 67/00, 67/033

Form PCT/ISA/210 (extra sheet) (July 1998)*